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<b>(54) Title:</b> PRIMERS FOR AMPLIFICATION OF BRCA1  <b>(57) Abstract</b>  Several portions of the intron regions surrounding exons (8, 15, 18, 20, 21 and 23) of BRCA1 and their adjacent intronic regions have been corrected. The corrections make it possible to construct oligonucleotide primers which are able to amplify the exons with greater fidelity than was previously possible, and which are particularly useful in amplifying sequences for CDGE to detect mutations in the BRCA1 gene.		

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## PRIMERS FOR AMPLIFICATION OF BRCA1

## BACKGROUND OF THE INVENTION

## 1. Field of the Invention

The invention relates to the BRCA1 gene. In particular, it relates to the nucleotide sequences of certain exons of the BRCA1 gene and the intronic regions adjacent thereto, and oligonucleotide primers for the amplification of the sequences.

## 2. Background Information

Breast cancer is the second most common cause of cancer related death among American woman. Atlanta: American Cancer Society, *Cancer Facts and Figures* (1995). It has been estimated that about 5-10% of breast cancer is due to an inherited mutation. Rowell, S., et al., *American Journal of Human Genetics* 55:861-865 (1994). Mutations in the BRCA1 gene are responsible for approximately 50% of early onset familial breast cancer. Easton et al., *American Journal of Human Genetics* 52:678-791 (1993) Located on chromosome 17q, BRCA1 is the first gene identified with a link to inherited breast and ovarian cancer. Miki et al., *Science* 266:66-71 (1994). Women from high risk families with a BRCA1 mutation have a 80-90% lifetime risk of breast cancer, a 40-65% lifetime risk of ovarian cancer, and an increased risk of colon cancer. Easton et al., *American Journal of Human Genetics* 52:678-701 (1993) Ford et al., *Lancet* 343:692-695 (1994). Male BRCA1 carriers have been shown to have an increased risk for prostate cancer and colon cancer, and in addition can pass

breast-ovarian cancer susceptibility to their daughters. Ford et al., *Lancet* 343:692-695 (1994).

Early detection of a BRCA1 mutation in an individual with a positive family history provides an opportunity for intervention to reduce the mortality associated with breast and ovarian cancer. Shattuck-Eidens et al., *Journal of the American Medical Association* 7:273:535-541 (1995)

Presymptomatic testing for individuals at high risk is made feasible through DNA-based genetic tests. Once identified, mutation-positive individuals have options for choosing preventative therapies against the long term affects of an inherited mutation.

The BRCA1 gene is approximately 100,000 base pairs of genomic DNA. Most of the 22 coding exons of BRCA1 span regions of between 100-500 basepairs, which in combination result in a protein of 1863 amino acids. Weber, B., *Scientific American* (January/February 1996). The exception, exon 11, containing approximately 3600 bp, makes up over half of the coding region of the gene.

Over 75% of BRCA1 mutations result from DNA alterations that shorten the protein product. Langston et al., *The New England Journal of Medicine* 334:137-142 (1996). However, all of the classic forms of mutations have been reported, including missense, a single nucleotide substitution that directs the incorporation of a different amino acid that does not affect the translation of the protein; nonsense, a nucleotide substitution that produces a signal to stop production of the protein; frameshift, the insertion or

deletion of one or more nucleotides that subsequently alters the production of the protein; and splice-site mutations, alterations in the intron/exon boundary that potentially cause the inclusion or exclusion of DNA in the coding sequence.

Detection of genetic mutations has been carried out by a variety of methods ranging from automated nucleotide sequencing, tests to observe the presence of a shortened gene product, and the analysis of specific BRCA1 mutations by way of probe-binding. Unfortunately, each test must be evaluated with regard to sensitivity, cost, and efficiency.

There are, however, nucleic acid-based screening tests that are cost effective and provide a rapid means of detecting mutations with maximal sensitivity. One technique in particular, constant denaturing gel electrophoresis (CDGE), a modification of denaturing gradient gel electrophoresis (DGGE), allows for an effective and accelerated investigation of the gene for the detection of known and unknown mutations. Hovig et al., *Mutation Research* 262:63-71 (1991). The cornerstone of this technique is a knowledge of the exact nucleotide sequence of the gene of interest.

Thus, in order to analyze BRCA1 using CDGE, it is imperative to know the intron sequence upstream and downstream of an exon to be investigated for possible mutations. Introns, located between each pair of exons, contain DNA that is transcribed into RNA, but is not used to synthesize the final protein product. Specific sequence information about an intron enables the construction of primers which can be used for Polymerase Chain Reaction (PCR) technology to produce

sufficient copies of a desired region of DNA for further analysis. These primers, short pieces of DNA that are complementary to the target sequence, are constructed in the intron approximately 100-150 base pairs upstream and downstream from the exon. The resulting PCR product embodies the intron sequence before the exon, the exon, and intron sequence after the exon.

Sequence information about BRCA1 has been published, and is available from the Breast Cancer Information Core Database (BIC). The sequence is listed on the World Wide Web for those members of the BIC. The BIC sequence of exon 20 and its upstream and downstream introns (SEQ ID NO:1) is shown in Table 2.

#### SUMMARY OF THE INVENTION

It is an object of this invention to provide compositions and methods for the detection of mutations to the BRCA1 gene in humans. In order to accomplish this object, the invention provides sequences and oligonucleotide primers for the amplification of portions of the BRCA1 gene using PCR technology. In one preferred aspect of the invention, these sequences relate to the regions of Exons 8, 15, 18, 20, 21 and 23 and their surrounding intronic regions (particularly to the region of exon 20).

Accordingly, it is an object of this invention to provide accurate sequences for exons 8, 15, 18, 20, 21 and 23 and for the intronic regions upstream and downstream from the exons. It is a particular object of the invention to provide

sequences to the aforementioned exons so that superior primers can be designed for the purpose of PCR amplification. The primers of the invention will be optimally suitable for amplifying portions of the BRCA1 gene for analysis using CDGE, in order to detect mutations in the BRCA1 gene. The primers will also be useful in other applications using PCR amplification. These applications include, but are not limited to, DNA sequencing, allele specific oligonucleotide assay, heteroduplex analysis, DNA chip technology and mismatch cleavage methods.

In accordance with this, it is a particular object of this invention to provide a forward primer capable of directing accurate amplification of a sequence of DNA which includes up to about 83 bases upstream to exon 20 along with exon 20. It is also a particular object of the invention to provide a reverse primer capable of directing accurate amplification of a sequence of DNA which includes up to about 83 bases downstream to exon 20 along with exon 20.

In addition, it is an object of the invention to provide forward and reverse primers for other exons of BRCA1, including exons 18, 21, and 23.

The term forward primer, according to the invention, is intended to mean a short complementary single stranded DNA sequence that binds to the DNA strand in the 5' to 3' direction and defines the upper boundaries of amplification. The term reverse primer, according to the invention, is intended to mean a short complementary single stranded DNA sequence that binds to the DNA strand in the 3' to 5'

direction and defines the lower boundaries of amplification. The primers are generally at least about 15 nucleotide bases in length, preferably at least about 20 nucleotide bases in length, and not more than about 65 bases in length (including a GC clamp, if present).

The term "isolated", in reference to a DNA sequence, is intended to mean a sequence which is at least free of its natural cellular environment.

"Exon 20 of the BRCA1 gene" is intended to mean SEQ ID NO:2, and any naturally occurring mutations and variants thereof. It is also intended to mean artificially constructed variants in which no more than two, preferably no more than one, base substitution has been made.

"Exon 21 of the BRCA1 gene" is intended to mean SEQ ID NO:38 and any naturally occurring mutations and variants thereof. It is also intended to mean artificially constructed variants in which no more than two, preferably no more than one, base substitution has been made.

Similarly, "Exon 8 of the BRCA1 gene", "Exon 15 of the BRCA1 gene", "Exon 22 of the BRCA1 gene" and "Exon 23 of the BRCA1 gene" are intended to mean SEQ ID NOS:48, 50, 52 and 53 and any naturally occurring mutations and variants thereof as well as artificially constructed variants in which no more than two, preferably no more than one, base substitution has been made.

The expression "substantially similar" as applied to a sequence is intended to include such naturally occurring mutations and variants and artificially constructed variants.



In reference to sequences, the expressions "95% (90%) identity" or "95% (90%) identical", according to the invention, are intended to include sequences in which no more than 5% (10%) of the bases are changed, preferably by substitution, as opposed to insertions or deletions.

The present invention utilizes CDGE to detect mutations in the BRCA1 gene. The BRCA1 genomic sequence information containing intron and exon regions around exon 20 was acquired from the Breast Cancer Information Core Database (BIC). The sequence is listed on the World Wide Web for members of the BIC. Exon 20 of BRCA 1

(5'CATGATTTTGAAGTCAGAGGAGATGTGGTCAATGGAAGAAACCACCAAGGTCCAAAGCGAGCAAGAGAATCCCAGGACAGAAAG-3') (SEQ ID NO: 2) was retrieved from the database along with its upstream and downstream introns. "Upstream" and "downstream" are descriptors for a location on a molecule of interest. Within the limits of this application, "upstream" refers to a position on a DNA sequence that is before (5') the exon of interest and "downstream" refers to a position on a DNA sequence that is after (3') the exon of interest. Upon inspection, the typed upstream intronic sequence in the BIC of exon 20 (SEQ ID NO:3) was missing nucleotides. These missing nucleotides are identified by the BIC in the typed sequence with the letter 'n'. These represent nucleotides which were not identified in the initial sequencing for various reasons including technology hindrance. Resolving these unknown nucleotides was essential for two reasons. First, the accurate melting profile of BRCA1 exon 20 was necessary for mutation detection using CDGE. Second,

given the best melting profile, the design of primers is facilitated.

Thus, there is a need in the art to identify the correct bases that occur in the intronic sequences adjoining exon 20 of BRCA1, as well as the other exons of BRCA1. There is also a need in the art to identify the accurate melting profiles of BRCA1 exon 20 and other exons of interest. There is also a need in the art to identify forward and reverse primers which are capable of accurately directing amplification of these exons. Identifying these needs and providing the correct sequence of bases for primer design is essential for sensitive mutation detection.

The term "mutation" according to the invention is intended to mean any alteration in the intronic or exonic sequences of BRCA1. Of particular interest are alterations to exonic sequences which are known or suspected to be associated with an increased risk of cancer by an individual, particularly of breast cancer.

The term "normal sequence", unless otherwise specified, is intended to mean a sequence which does not contain such mutations. This may also be referred to as "wild-type sequence". It is noted that certain variants in the exonic or intronic sequences of BRCA1 occur which are not known to confer increased susceptibility to cancer. For example, single base substitutions which do not result in an alteration to the protein produced (normal polymorphisms) are known to occur. While the invention is particularly intended to detect mutations which are known or suspected to be associated with

increased cancer risk, it can also be used to detect these variants.

It will be understood by persons of skill in the art that the term "sequence" is intended to include single- and double-stranded forms as well as sequences which are complementary thereto.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Schematic Diagram of the Protocols of the Invention

Figure 2. Melt profile of Exon 20 obtained using the primers of the invention

Figure 3. Melt profile of Exon 20 obtained using primers MY-20F (SEQ ID NO:39) and MY-20R (SEQ ID NO:40) taken from the BIC sequence.

Figure 4. Superimposed profiles of Figures 2 and 3

Figure 5. BRCA1 Exon 21 wild-type and mutant PCR products.

Samples are arranged on the gel in the following order:

- A. 100 basepair DNA ladder
- B. wild-type sample
- C. wild-type sample
- D. wild-type sample
- E. negative control
- F. blank
- G. 5438insC

- H. 5438insC
- I. 5438insC
- J. negative control

Figure 6. Perpendicular Denaturing Gradient Gel Electrophoresis (DGGE).

Figure 7. Schematic Diagram of Denaturing Gradient Gel Electrophoresis. The method is performed as detailed by Anne-Lisa Borresen ("Constant Denaturing Gel Electrophoresis (CDGE) in Mutation Screening" in *Technologies for Detection of DNA Damage and Mutations* (Ed. G.D. Pfeifer) Chapter 11.22., 1995).

Figure 8. Constant Denaturing Gel Electrophoresis (CDGE)  
Samples are arranged on the gel in the following order (left to right):

- A. 100 base pair ladder
- B. wild-type (homoduplex)
- C. 5438insC (heteroduplex)
- D. wild-type (homoduplex)
- E. 5438insC (heteroduplex)
- F. wild-type (homoduplex)
- G. 5438insC (heteroduplex)
- H. wild-type (homoduplex)
- I. 5438insC (heteroduplex)
- J. wild-type (homoduplex)
- K. 5438insC (heteroduplex)

- L. unrelated
- M. unrelated
- N. unrelated
- O. unrelated

Figure 9. Schematic Diagram of Constant Denaturing Gel Electrophoresis. Method performed as detailed by Anne-Lisa Borresen, cited above.

#### DETAILED DESCRIPTION OF THE INVENTION

The strategy used to accomplish the objectives and goals of the invention involved two procedures:

- I. Optimization of a BRCA1 exon using CDGE mutation analysis.
  1. Determination of the correct normal sequence of a selected exon of BRCA1 (e.g. exon 20) and its adjacent introns within about 83 base pairs.
  2. Determination of the melting profile of the intron/exon sequence.
  3. Design of primers which operate in the upstream and downstream introns for optimal amplification of the exon including the splice sites.
  4. Extraction of DNA from white blood cells of a sample for use as a wild-type control.
  5. Amplifying the exon of interest from a wild-type control by way of the polymerase chain reaction using primers of the invention.
  6. Determination of the optimal running conditions for a

constant denaturing gel by running a denaturing gradient gel using the amplification products of a wild-type control sample.

7. Mutation analysis on a constant denaturing gradient gel (CDGE) of a wild-type control sample.

II. Standard strategy for CDGE mutation analysis of BRCA1 exon.

1. Extraction of DNA from white blood cells of a patient sample.

2. Amplifying the exon of interest from a patient sample by way of the polymerase chain reaction using the primers of the invention.

3. Mutation analysis on a constant denaturing gradient gel (CDGE) of a patient sample.

It is noted that for convenience, intronic bases are generally shown throughout the application in lower case, whereas exons are indicated in upper case. It is also noted that where dots, dashes or asterisks (\*) are inserted into sequences, it is for the purposes of alignment, or to indicate the absence of a particular base which is present in a corresponding sequence. IUB codes have been used in certain locations to designate bases, as will be familiar to those skilled in the art. (Briefly, R designates A or G; Y, C or T; K, G or T; M, A or C; S, G or C; W, A or T; and N, any base.)

In the description which follows, the methods of the invention are exemplified using Exons 20 and 21 as working

examples; however, the techniques will be suitable for use on other intron/exon regions of BRCA1, as will be appreciated by persons of skill in the art.

#### Example 1

##### Determination of DNA sequence of Exon 20

Determination of the DNA sequence of Exon 20 and adjacent introns was done by performing fluorescent base sequencing of approximately 290 base pairs of PCR product encompassing Exon 20 and adjacent introns. Genomic DNA was isolated from white blood cells of subjects with a family history of breast cancer using the Blood and Cell Culture DNA Maxi Kit (Qiagen, Germany). The fact that most breast cancer patients are negative with respect to mutations on BRCA1 made it possible to use patient samples for the determination of the correct normal sequence. Samples for the sequencing were selected from 10 patients who were determined to be of normal genetic makeup with respect to Exon 20.

Dideoxy sequence analysis was performed following polymerase chain reaction amplification of BRCA1 exon 20.

Exon 20 of the BRCA1 gene was subjected to cycle sequencing with dye-labeled dideoxynucleotides, AmpliTaq<sup>®</sup> DNA Polymerase, FS (Roche Molecular Systems, Branchburg, NJ, USA) and primers for amplification.

In this reaction, each growing chain is simultaneously terminated and labeled with a dye that corresponds to a particular base (Comparative PCR Sequencing: A Guide to Sequencing-Based Mutation Detection, The Perkin-Elmer

Corporation, Applied Biosystems Division, 1995).

The primers used for the PCR amplification are suitable for the cycle sequencing reactions. Accordingly, the following primers for BRCA1 exon 20 were used:

forward primer: 5'- ATA TGA CGT GTC TGC TCC AC -3' []

reverse primer: 5'- GGG AAT CCA AAT TAC ACA GC -3' []

Table 1: PCR PROTOCOL

BRCA1 Exon 20 PCR Conditions:

1X PCR Buffer (Roche Molecular Systems, Branchburg, NJ, USA)  
200  $\mu$ M deoxynucleotides  
0.4  $\mu$ M forward primer  
0.4  $\mu$ M reverse primer  
2.5 units Amplitaq<sup>®</sup> DNA polymerase (Roche Molecular Systems, Branchburg, NJ, USA)  
1.4 mM MgCl<sub>2</sub>  
100 ng DNA template  
Add up to 25 microliters deionized water

BRCA1 Thermocycling Conditions:

PCR Machine: PTC 100 Programmable Thermal Controller, MJ Research, Inc.

1. Denature double stranded PCR product with an initial step at 94° for 4 minutes
2. 35 cycles of the following:
  - 94°C 30 seconds (denaturing)
  - 55°C 1 minute (annealing)
  - 72°C 2 minutes (extension)
3. Final step:
  - 72°C 5 minutes

Fluorescent dye was attached to the PCR product for automated sequencing using the Dye Terminator Cycle Sequence Ready Reaction Kit (Perkin-Elmer<sup>®</sup> cat# 402122). DNA sequencing was performed in both forward and reverse directions on an Applied Biosystems, Inc. (ABI) automated sequencer (Model 377). The software used for analysis of the resulting data



was "Sequence Navigator" purchased through ABI.

The correct sequence was determined by comparing the BIC sequence to sequences generated from 7 patients at OncorMed, Inc. The sequence obtained is shown in SEQ ID NO:4. The sequence differs from the sequence as published by BIC with respect to the following.

- 1). BIC Sequence: CAC TCCATTG (SEQ ID NO:5)  
Sequence of the invention: T TCCATTG  
location: ~55bp from exon
- 2). BIC Sequence: AAGGA\*GCTTCT (SEQ ID NO:6)  
Sequence of the invention: AAGGAAGCTTCT (SEQ ID NO:7)  
location: -45bp from exon
- 3). BIC Sequence: GT\*GTGN\*TG\*TT (SEQ ID NO:8)  
Sequence of the invention: GTTGTGTTTGGTT (SEQ ID NO:9)  
location: ~19bp from exon

Thus, the BIC sequence was determined to have a number of missing or incorrect bases, specifically in the upstream intron. The sequence determined for the 83 bases immediately upstream of Exon 20 are shown in SEQ ID NO:10.

The sequences of Exons 8, 15, 18, 21 and 23 were determined using similar methods.

#### Exon 8

The following differences were seen in the sequence of

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Sequence of the invention: AAGGAAGCTTCT (SEQ ID NO:7)  
location: ~45bp from exon
- 3). BIC Sequence: GT\*GTGN\*TG\*TT (SEQ ID NO:8)  
Sequence of the invention: GTTGTGTTTGGTT (SEQ ID NO:9)  
location: ~19bp from exon

Thus, the BIC sequence was determined to have a number of missing or incorrect bases, specifically in the upstream intron. The sequence determined for the 83 bases immediately upstream of Exon 20 are shown in SEQ ID NO:10.

The sequences of Exons 8, 15, 18, 21 and 23 were determined using similar methods.

#### Exon 8

The following differences were seen in the sequence of

Exon 8 and its downstream intronic sequence:

BIC sequence (first 51 bp of exon 8):

C AGGAAACCAr TCTCAGTGTC CCAACTCTCT AACCTTGAA  
CTTGTGAGAA

(SEQ ID NO:11)

Sequence of the invention:

C AGGAAACCAG TCTCAGTGT\* CCAACTCTCT AACCTTGAA  
CT\*GTGAGAA

(SEQ ID NO:12)

Thus, one each of bases C and T have been removed and r  
identified as G at the indicated positions in Exon 8.

BIC sequence (beginning 64 bp downstream of Exon 8):

gccamaaatc (SEQ ID NO:13)

Sequence of the invention:

gccaaaaatc (SEQ ID NO:14)

Thus, "m" has been identified as "a" at the indicated  
position.

#### Exon 15

The following differences were seen in the intronic sequences  
of Exon 15.

BIC sequence (beginning ~60 bp upstream of Exon 15):

gtatgaaatg tcctntcaca aggggtggcg (SEQ ID NO:15)

Sequence of the invention:

gtatgatttg tcctttcaca attggtggcg (SEQ ID NO:16)

BIC sequence (beginning ~20 bp downstream of Exon 15):

attggarcam acactytgat (SEQ ID NO:17)

Sequence of the invention:

attggaacaa acactttgat (SEQ ID NO:18)

#### Exon 18

The following differences were seen in the intronic sequence to Exon 18:

BIC sequence (beginning ~20 bp upstream):

g agtgtktctc attctgcag (SEQ ID NO:19)

Sequence of the invention:

g agtggttttc attctgcag (SEQ ID NO:20)

"k" and "c" have each been changed to "t", as indicated.

BIC sequence (beginning ~91 bp downstream):

ttgctgatgc tgagtcvvvv gctgatgctt gagtctgagt cncnaaagnc  
ctttaattgt aatactaact (SEQ ID NO:21)

Sequence of the invention:

ttgctgatgc tgagctgag ttaccaaaggt ctttaattgt aatactaaact (SEQ  
ID NO:22)

Thus, in the downstream intronic region the 34 base sequence "vvvv gctgatgctt gagtctgagt cncnaaagnc" (SEQ ID NO:23) is changed to the 16 base sequence "tgag ttaccaaaggt" (SEQ ID NO:24), as well as the insertion of an additional "a".

#### Exon 21

The following differences were seen in the Exon 21 region.

The intronic sequence for the 81 bp immediately upstream to Exon 21 was found to be:

gaataaaagc caatattctt ttataactag attttccttc tctccattcc  
cctgtccctc tctcttcctc tcttcttcca g (SEQ ID NO:25)

which does not correspond closely to the published BIC sequence in this region.

BIC sequence (downstream 10 bp from Exon 21):

gcctcgggag (SEQ ID NO:26)

Sequence of the invention:

gcct\*gggag

BIC sequence (downstream 18 bp from Exon 21):

gaan\*ccag

Sequence of the invention:

gaacccag

BIC sequence (downstream 78 bp from Exon 21):

caaggtccc

Sequence of the invention:

caagatccc

### Exon 23

The following changes were seen in the sequence to Exon 23:

BIC sequence (beginning ~11 bp upstream of Exon 23

tgggganccag\*GTGTCC (SEQ ID NO:27)

Sequence of the invention:

tggggatccagGGTGTCC (SEQ ID NO:28)

Thus, a "G" is present at the beginning of the exon which was not contained in the BIC exon sequence, and a "t" was not identified in the BIC sequence.

The published BIC sequence also failed to identify the following 53 bp sequence directly downstream of Exon 23:

gtaaggtgcctgcatgtacctgtgctatatggggtccttttgcacgggtttgg  
(SEQ ID NO:29)

According to the invention, this sequence directly follows the exon.

In addition, at a location about 104 bp downstream of the exon (corresponding to 51 bp downstream in the BIC sequence), the BIC sequence

tttttaaata (SEQ ID NO:30)

is replaced by

tttt\*aaata

in the invention.

#### Example 2

Determination of the melting profile of the intron/exon sequence.

Theoretical analysis of the exon with flanking intron is performed prior to running samples through the CDGE system. Initial optimization is critical for maintaining the highest



degree of sensitivity possible. The sequence is entered into computer software created to analyze the melting profile of an exon. The software was designed by Bio-Rad Laboratories, Hercules, California, titled, *MacMelt Software - DNA Melt Profile Macintosh Software for the D GENE System Version 1.0*. The term melting profile refers to the change in structure of the PCR product when it transitions from a double-stranded molecule to a single-stranded molecule. The melting profile is characteristic of the DNA sequence residing in a particular PCR product and is demonstrated visually on the computer software by way of a graph. The profile theoretically predicts regions where base changes, differing from the original sequence, can be detected. Thus, one nucleotide change will create a different curve on the graph. The altered curve is compared to a "normal" or wild-type curve on the same graph. If it is possible to resolve the altered curve from the normal curve, then it is possible to resolve the two different sequences on a polyacrylamide gel with a constant denaturant (CDGE). In summary, knowing the correct nucleotide sequence of the exon facilitates the construction of a melting profile that clearly differentiates between a normal sequence and a mutant sequence. Once this is established, PCR products from human samples with unknown DNA alterations are run on the constant denaturing gel. The PCR product, processed from genomic DNA, migrates to a certain position, in a defined time period, on the gradient gel. This position is based solely on the sequence of the PCR product. If one base change has occurred, the band will migrate to a

different position when compared to a normal control.

Examples of this technology are shown in Figures 2 and 3. Figure 2 illustrates the melt profile for Exon 20 obtained using the primers of the invention. The y-axis is in units of °C and the x-axis defines the basepairs. The highest domain on the graph is a result of the GC clamp primer. The exon spans a region between 124 basepairs and 207 basepairs. This is an optimal melt profile for two reasons. First, there are not any significant deviations from the 72°C mark. Deviations would include inclines or declines along the length of the profile. These inclines or declines represent higher and lower melting domains in the sequence. For every significant change in a melting domain, a control sample that has a single base change in that area must be run through the system to ensure sensitivity of strand separation. Second, the entire profile melts in a staircase fashion. There is sequential melting from the upstream (GC-clamp region) portion of the exon to the downstream except for small deviations that are less than 0.25°C (these can be seen when the melt profile is magnified). These very small deviations in temperature are negligible. The staircase fashion melt profile is important because higher domains at the downstream end of the sequence might not be detected if the upstream portion denatures first in the electrophoresis system.

The melt sequence for Figure 3 incorporates primers taken from the BIC sequence referred to as MY-20F (SEQ ID NO:39) and MY-20R (SEQ ID NO:40). The sequence includes the MY-20F (SEQ ID NO:39) primer, the intron sequence following the primer,

Exon 20, intron sequence before the MY-20R primer and the MY-20R primer (SEQ ID NO:40). The exon spans a region between 121 basepairs and 204 basepairs. Although this sequence is very similar to the sequence used in Figure 2, it gives a very different melt profile. (The two profiles are superimposed in Figure 4 for the purpose of comparison.) There are inclines and declines occurring throughout the profile. In order to obtain the highest sensitivity possible, each variation would need a different control. Instead of having one or two controls, (i.e. Figure 2), an extensive control panel made up of several mutants would have to be produced and examined for each gel run. This would turn what should be a straightforward mutation analysis procedure into a time consuming task with diminished sensitivity. In contrast, the present invention results in a straightforward efficient procedure which produces superior results.

### Example 3

#### Design of Primers

The primers were designed using the primer analysis software, Oligo 4.03 developed by National Biosciences, Inc., Plymouth, MN (copyright 1992 Wojciech Rychlik).

The bases making up potential primers were analyzed in the Oligo computer program. The analysis procedures indicate problem areas inherent within the short sequence of bases that would inhibit the PCR reaction. Primers determine the success or failure of a PCR amplification (Erlich, Henry A., *PCR Technology, Principles and Application for DNA Amplification*,

Stockton Press, 1989). Problems can arise because of primer characteristics such as (1) stretches of a single nucleotide sequence (e.g. -TTTT-), (2) secondary structure (e.g. hairpin loops), (3) primers which are complementary to each other or to themselves, and other characteristics which would affect the efficiency of the PCR amplification process. In addition, it is important that a primer amplify a DNA sequence which includes the splice sites of the exon in order to be certain that errors in the final protein product do not occur due to premature termination of transcription or other problems in initiating and terminating transcription.

A forward primer was developed 83 basepairs before the exon and a reverse primer was developed 63 base pairs after the exon:

Forward primer: 5'- TAAATATGACGTGTCTGCTC -3' (SEQ ID NO:31)

Forward primer with a CG clamp-: 5'-

CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGTAAATA

TGACGTGTCTGCTC -3' (SEQ ID NO:32)

Reverse primer: 5'- TGAAGCGGCCCATCTCTGCA -3' (SEQ ID NO:33)

The forward primer (SEQ ID NO:31) is 20 bases in length, and has optionally attached at the 5' end a further GC clamp of 40 bases, as shown in SEQ ID NO:32. It will be appreciated by the skilled artisan that a "GC clamp" is a nucleotide sequence containing optionally one thymidine base and otherwise guanine and cytosine bases, which is attached to a primer at the 5' end or 3' end for the purpose of affecting the melt profile of the primer during electrophoresis. At the

end of the clamp, 20-25 nucleotides of "unique" sequence is attached. The GC clamp contributes a non-melting region to the PCR product. This is useful because a PCR product without a GC clamp has its own melting characteristics. For example, a GC rich area in the center of a sequence of interest will create a higher melting domain in that region or an arrangement of bases creating several melting variations. Melting variations in the sequence make it difficult to detect mutations in some areas. The attachment of a GC clamp on one of the ends absolves melting variants and creates a profile that is more sensitive for mutation detection.

In addition, the GC-clamp enables staining the gel with ethidium bromide or SYBR® green I nucleic acid stain (Molecular Probes, Inc.) and viewing the migration of the PCR product under a UV light.

The PCR primers are useful for amplification and subsequent analysis of the PCR product on a constant denaturing gradient gel (CDGE). The PCR primers may also be labeled with a radiolabel, a fluorescent label, a bioluminescent label, a chemiluminescent label, or an enzyme label, using means familiar to those of skill in the art.

#### Example 4

##### Polymerase Chain Reaction (PCR) Amplification

To test individual subjects for mutations at Exon 20 of the BRCA1 gene, the primers of the invention are used in PCR amplification. Genomic DNA (100 nanograms) extracted from white blood cells of a subject was amplified in a final volume

of 50 microliters containing 100 nanograms genomic DNA, 1X PCR buffer (Roche Molecular Systems, Branchburg, NJ, USA), 200 micromolar dNTP mix, 1 mM MgCl<sub>2</sub>, 0.2 micromolar forward primer (designated BRCA1-20-F, SEQ ID NO:32), 0.3 micromolar reverse primer (designated BRCA1-20-R, SEQ ID NO:33), 2.5 units AmpliTaq DNA polymerase (Roche Molecular Systems, Branchburg, NJ, USA), and deionized water up to 50  $\mu$ l.

The primers were synthesized at Bioserve Biotechnologies in Laurel, MD using standard 0.2 $\mu$ M synthesis scale. Thirty-five cycles were performed, each consisting of denaturing (95°C; 1 minute), annealing (55°C; 1 minute), and extension (72°C; 1.5 minute), except during the first cycle in which the denaturing time was 3 minutes. In addition, following the thirty five cycles, there was a 10 minute extension at 72°C, a three minute denaturation at 94°C and a 1 hour heteroduplexing step at 65°C.

PCR products were purified using an ethanol precipitation protocol. At room temperature the following reagents were added, in order, into a 1.5ml Eppendorf tube: 30 $\mu$ l 3M sodium acetate (Sigma: St. Louis, MO), 2 $\mu$ l 20mg/ml glycogen (Boehringer Mannheim: Indianapolis, IN), 45 $\mu$ l of PCR product, and 223 $\mu$ l of deionized water. After mixing these components, 600 $\mu$ l of 100% EtOH was added. The reaction was left at room temperature for 15 minutes and then placed in a centrifuge at 13,000 rpm for 15 minutes. After the spin, the supernatant was drained, leaving a small pellet at the bottom of the microfuge tube. One milliliter of cold 70% ethanol was added to the tube. The reaction was placed in the centrifuge and

spun at 13,000 rpm for 5 minutes. The liquid was drained, leaving a small pellet on the bottom of the centrifuge. The sample was dried in a speed-vac for 15 minutes. The dried pellet was diluted in 10 $\mu$ l of deionized water. 10% of the purified PCR product was run on a 2% TAE agarose gel for confirmation that the correct PCR product was produced.

#### Example 5

##### BRCA1 Exon 21 Wild-type and mutant PCR products

PCR products for Exon 21 (SEQ ID NO:38) were obtained as described above, using forward and reverse primers for exon 21 (SEQ ID NO:41) and (SEQ ID NO:42), which were developed and constructed as described for Exon 20.

Six PCR products from wild-type and mutant samples were run on a 7.5% polyacrylamide gel for 30 minutes at 200V on a Bio-Rad Mini-Protean II Electrophoresis Cell (Bio-Rad: Hercules, CA). Template controls without DNA were processed concurrent to the mutant and wild-type samples and run on the gel to determine the presence of contaminants in the stock reagents. The samples were sized using a 100 bp DNA ladder (Gibco BRL: Gaithersburg, MD), to confirm the presence and quality of the products. The results are shown in Figure 5.

#### Example 6

##### Perpendicular Denaturing Gradient Gel Electrophoresis (DGGE)

Perpendicular DGGE was used to determine the number of melting domains and optimal denaturing conditions for mutant

and wild-type PCR products from exon 21 of BRCA1, as shown in Figure 6. Using the D-Gene Denaturing Gel Electrophoresis System (Bio-Rad: Hercules, CA), mutant and wild-type samples were mixed and loaded into a single well that extended across the top of the gel. The gel was run submerged in 1X TAE buffer (40mM Tris/20mM acetic acid/1mM EDTA) (Bio-Rad: Hercules, CA) at 56°C for three hours at a constant 130V (Figure 6). The denaturant, composed of varying concentrations of 7M urea and formamide, ranged from 20% to 60% (left to right). After electrophoresis, the gel was stained with SYBR® green I (Molecular Probes, Inc.: Eugene, OR) and viewed under a UV transilluminator. Using the gel image of DGGE, the single denaturing condition for constant denaturing gel electrophoresis (CDGE) was determined to be 49%. This value is calculated at the point on the gel where wild-type and mutant products are maximally separated. After this initial optimization, every sample from exon 21 of BRCA1 was analyzed at 49%.

During electrophoresis on the denaturing gradient gel, mutant and wild-type samples migrate vertically through an electrophoretic field encountering a denaturing environment that is created horizontally across the gel. At low denaturant concentrations, double stranded DNA undergoes partial melting. Higher denaturants melt the PCR products even further, causing greater changes in conformation and migration rates. Due to the gradient difference across the gel, PCR products take the shape of an S-curve (refer to Figure 7). This curve is formed because of the differences in



migration rates of double stranded, branched and single stranded DNA molecules. The greater the double stranded nature of the molecule, the faster it will move through the porous matrix of the polyacrylamide gel.

#### Example 7

Mutation analysis on a constant denaturing gradient gel (CDGE) using the PCR product.

A mutation analysis was performed on the PCR products obtained in Example 4 using CDGE. The methods used in the analysis are described in Chapter II.22 (Constant Denaturant Gel Electrophoresis (CDGE) in Mutation Screening) of *Technologies for detection of DNA Damage and Mutations* (Plenum Press, 1995, ed. G.P. Pfeifer).

BRCA1 exon 21 5438insC mutant (Castilla, L.H. et al., 1994, *Nature Genetics* 8:387-391) and wild-type PCR products were run on a constant denaturing gel for 3.5 hours at 130V and submerged in 1X TAE buffer that was heated to 56°C (Figure 8). Mutant and wild-type samples were screened using a 49% denaturing gel. The denaturant was a combination of 12.6 mL of 70% denaturant and 5.4 mL of 0% denaturant to obtain a final volume of 18 mL of 49% denaturant (100% denaturant corresponds to 7M urea and 40% formamide). After electrophoresis, the gel was stained with SYBR® green I (Molecular Probes, Inc.: Eugene, OR) and viewed under a UV transilluminator.

Prior to the gel run, the samples were heat-treated at 94°C for 3 minutes to melt the PCR products and allowed to

reanneal at 65°C for 1 hour to create heteroduplexes and homoduplexes. By definition, a heteroduplex is a double stranded nucleic acid in which each strand is amplified from a different template and not exactly complementary. The term complementary describes the nature of the four bases constituting the DNA double helix (adenine, thymine, cytosine and guanine) and their chemical interaction with one another so that adenine always binds with thymine and guanine always binds with cytosine. In the context of the present specification, a heteroduplex has at least one base pair that does not uphold the normal nucleotide interaction stated above. Homoduplexes have complementary PCR products generated from the same template.

The example in Figure 8 demonstrates the use of CDGE for mutation detection using amplified PCR product from BRCA1 exon 21. The target DNA used for this experiment was human DNA from a patient sample without a history of inherited breast cancer and human DNA known to have an insertion of a cytosine at nucleotide 5438. The PCR products from these examples are representative of both alleles of exon 21 BRCA1. Therefore, if one allele is normal and one allele has a mutation, then 50% of the PCR product will be amplified from the normal allele and 50% of the PCR product will be amplified from the mutant allele.

The formation of heteroduplexes and homoduplexes made during the melting and reannealing steps allows for a graphical display of the PCR products. Wild-type homoduplexes and mutant homoduplexes are represented on the gel by a single

band. These bands will migrate to a specific point on the gel in a designated period of time. The migration pattern is sequence dependent, therefore the position for each homoduplex band will be different. Heteroduplexes have different pattern bands due to the combination of mutant and wild-type polynucleotide strands (refer to Figure 6).

The example of BRCA1 exon 21 shows the heteroduplex bands above the homoduplexes. This occurs because the heteroduplex melts sooner than the homoduplex and migrates at a slower rate.

A schematic diagram illustrating the protocol for CDGE is shown in Figure 9.

A listing of DNA sequences described herein, including sequences of the invention, is presented in Table 2.

**Table 2****SEQUENCES DISCLOSED IN THE APPLICATION**

**SEQ ID NO:1. Exon 20 from the BIC database with upstream and downstream intronic regions**

```

      tg caccccaaga cggatggggnn ctcccccncc nctatatttga
tttagaaant gggttcccat gngtaagcng tgcgaccctt gcctcaatga
ctnctcctaa tctcccaaata tctaggatag ngggtgancc ctcnctgnc
tgatccntaa atatgacgtg tctgctccac cactccattg aaggagcttct
ctttctctt atcctgatgg gtgtgntgtt tctttcagCA TGATTTTGAA
GTCAGAGGAG ATGTGGTCAA TGGAAGAAAC CACCAAGGTC CAAAGCGAGC
AAGAGAATCC CAGGACAGAA AGgtaaagct cctccctca agttgacaaa
aatctcacc caccactctg tattccactc ccctttgcag agatgggccc
cttcattttg taagacttat tacatacata cacagtgcta gatactttca
cacaggttct tttttcactc ttccatccca accacataaa taagtattgt
ctctacttta tgaatgataa aactaagaga tttagagagg ctgtgtaatt
tggaattcccg tctcgggttc agatc

```

**SEQ ID NO:2 Exon 20 of BRCA1 (BIC)**

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CATGATTTTGAAGTCAGAGGAGATGTGGTCAATGGAAGAAACCACCAAGGTCCAAAGCGAGC
AAGAGAATCCCAGGACAGAAAG

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**SEQ ID NO:3 Upstream intronic sequence of Exon 20 in the BIC**

```

      tg caccccaaga cggatggggnn ctcccccncc nctatatttga
tttagaaant gggttcccat gngtaagcng tgcgaccctt gcctcaatga
ctnctcctaa tctcccaaata tctaggatag ngggtgancc ctcnctgnc
tgatccntaa atatgacgtg tctgctccac cactccattg aaggagcttct
ctttctctt atcctgatgg gtgtgntgtt tctttcag

```

SEQ ID NO:4 Exon 20 with upstream and downstream intronic regions according to the invention

tg caccccaaga cggatggggn ctccccncc nctattttga  
tttagaaant gggttcccat gngtaagcng tgcgaccctt gcctcaatga  
ctnctcctaa tctcccaaatt tctaggatag ngggtgancc ctccnctgnc  
tgatccntaa atatgacgtg tctgctccac ttccattg aaggaagcttct  
ctttctctt atcctgatgg gttgtgtttggtt tctttcag  
CATGATTTTGAA GTCAGAGGAG ATGTGGTCAA TGGAAGAAAC CACCAAGGTC  
CAAAGCGAGC AAGAGAATCC CAGGACAGAA AGgtaaagct ccctccctca  
agttgacaaa aatctcacc caccactctg tattccactc ccctttgcag  
agatggggccg cttcattttg taagacttat tacatacata cacagtgcata  
gatactttca cacaggttct tttttcactc ttccatccca accacataaa  
taagtattgt ctctacttta tgaatgataa aactaagaga tttagagagg  
ctgtgtaatt tggattcccg tctcgggttc agatc

SEQ ID NO:5 BIC Sequence ~55bp upstream from exon 20:  
CAC TCCATTG

SEQ ID NO:6 BIC Sequence ~45bp upstream from exon 20:  
AAGGA\*GCTTCT

SEQ ID NO:7 Sequence of the invention located ~45bp upstream  
of exon 20:  
AAGGAAGCTTCT

SEQ ID NO:8 BIC Sequence ~19bp upstream from exon 20:  
GT\*GTGN\*TG\*TT

SEQ ID NO:9 Sequence of the invention located ~19bp upstream of exon 20:

GTTGTGTTTGGTT

SEQ ID NO:10 Intronic sequence of the invention upstream of exon 20:

taa atatgacgtg tctgctccac ttccattg aaggaagcttct ctttctctt  
atcctgatgg gttgtgtttgggtt tctttcag

SEQ ID NO:11 BIC Sequence first 51 bp of exon 8:

C AGGAAACCAr TCTCAGTGTC CCAACTCTCT AACCTTGGAA CTTGTGAGAA

SEQ ID NO:12 Sequence of the invention, first 51 bp of exon 8:

C AGGAAACCAG TCTCAGTGT\* CCAACTCTCT AACCTTGGAA CT\*GTGAGAA

SEQ ID NO:13 BIC sequence beginning 64 bp downstream of exon 8:

gccamaaatc

SEQ ID NO:14 Sequence of the invention beginning 64 bp downstream of exon 8:

gccaaaaatc

SEQ ID NO:15 BIC sequence beginning ~60 bp upstream of exon 15:  
gtatgaaatg tcctntcaca aggggtggcg

SEQ ID NO:16 Sequence of the invention beginning ~60 bp

upsteam of exon 15:

gtatgatttg tcctttcaca attggtggcg

SEQ ID NO:17 BIC sequence beginning ~20 bp downstream of exon 15:

attggarcam acactytgat

SEQ ID NO:18 Sequence of the invention beginning ~20 bp downstream of exon 15:

attggaacaa acactttgat

SEQ ID NO:19 BIC sequence beginning ~20 bp upstream of exon 18:

g agtgtktctc attctgcag

SEQ ID NO:20 Sequence of the invention beginning ~20 bp upstream of exon 18:

g agtggttttc attctgcag

SEQ ID NO:21 BIC sequence beginning ~91 bp downstream of exon 18:

ttgctgatgc tgagtcvvvv gctgatgctt gagtctgagt cncnaaagnc  
ctttaattgt aataactaact

SEQ ID NO:22 Sequence of the invention beginning ~91 bp downstream of exon 18:

ttgctgatgc tgagtctgag ttaccaaagggt ctttaattgt aataactaaact

SEQ ID NO:23 Variant portion of SEQ ID NO:21:

vvvv gctgatgctt gagtctgagt cncnaaagnc

SEQ ID NO:24 Sequence of the invention corresponding to SEQ ID NO:23

tgag ttaccaaaaggt

SEQ ID NO:25 Intronic sequence of the invention upstream and adjacent to exon 21:

gaataaaagc caatattctt ttataactag attttccttc tctccattcc  
cctgtccctc tctcttcctc tcttcttcca g

SEQ ID NO:26 BIC sequence downstream 10 bp from exon 21:

gcctcgggag

SEQ ID NO:27 BIC sequence beginning ~11 bp upstream of exon 23:

tgggganccag\*GTGTCC

SEQ ID NO:28 Sequence of the invention beginning ~11 bp upstream of exon 23:

tggggatccagGGTGTCC

SEQ ID NO:29 53 bp sequence of the invention directly downstream of exon 23:

gtaaggtgcctgcatgtacctgtgctatatggggtccttttgcattgggtttgg



SEQ ID NO:30 BIC sequence 51 bp downstream of exon 23:

tttttaaata

SEQ ID NO:31 Forward primer of exon 20 of the invention

TAAATATGACGTGTCTGCTC

SEQ ID NO:32 Forward primer with GC clamp for exon 20 of the invention

CGCCCCCGCGCCCCCGCGCCCGTCCCGCCGCCCCCGCC

CGTAAATATGACGTGTCTGCTC

SEQ ID NO:33 Reverse primer for exon 20 of the invention:

TGAAGCGGCCCATCTCTGCA

SEQ ID NO:34 Intronic sequence of the invention located  
~63bp upstream and adjacent to exon 20:

CACTTCCATTGAAGGAAGCTTCTCTTTCTCTTATCCTGATGGGTTGTGTTTGGTTTCTTTCA  
G

SEQ ID NO:35 Intronic sequence of the invention located  
downstream and adjacent to exon 20:

gtaaagct ccctccctca agttgacaaa aatctcaccc caccactctg  
tattccactc  
ccctttgcag agatgggccg cttca

SEQ ID NO:36 GC Clamp, forward primer and exon 20

CGCCCCCGCGCCCCCGCGCCCGTCCCGCCGCCCCCGCCCGTAAATATGACGTGTCTGCTCCA  
CTTCCATTGAAGGAAGCTTCTCTTTCTCTTATCCTGATGGGTTGTGTTTGGTTTCTTTCAGC

ATGATTTTGAAGTCAGAGGAGATGTGGTCAATGGAAGAAACCACCAAGGTCCAAAGCGAGCA  
AGAGAATCCCAGGACAGAAAGGTAAAGCTCCCTCCCTCAAGTTGACAAAAATCTCACCCCAC  
CACTCTGTATTCCACTCCCCTTTGCAGAGATGGGCCGCTTCA

SEQ ID NO:37 GC Clamp:

CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG

SEQ ID NO:38 Exon 21

ATCTT CAGGGGGCTA GAAATCTGTT GCTATGGGCC CTTACCAAC ATGCCACAG

SEQ ID NO:39 MY-20F

atatgacgtgtctgctccac

SEQ ID NO:40 MY-20R

agtcttacaaaatgaagcgg

SEQ ID NO:41 Forward Primer of the invention for exon 21

CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGTATAACTAGATTTTCCTTCT

SEQ ID NO:42 Reverse Primer of the invention for exon 21

CTCCACTATGTAAGACAA

SEQ ID NO:43 Forward Primer of the invention for exon 18

TTCAACTTCTAATCCTTT

SEQ ID NO:44 Reverse Primer of the invention for exon 18

CCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGTGAGGTGTTAAAGGGAGGA

SEQ ID NO:45 Forward Primer of the invention for exon 23

CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCGCCCCGTGATGAAGTGACAGTTCCAG

SEQ ID NO:46 Reverse Primer of the invention for exon 23

GTGATAAACCAAACCCATGC

SEQ ID NO:47 Exon 8 and adjacent intronic sequence (BIC)

gaaaaattta agacaacmsa araaaaawy cmaatcacam atatcccaca  
cattttatta ttmctmctmc wattattttg wagagmctgg gtctcacycy  
kttgctwatg ctggtctttg aacyccykgc cycaarcart cctstccab  
cctcccaarg tgctggggat wataggcatg arctaccgc tcccagccm  
agacaatttt agtgtwgca aaattcctgg gcattttttc maggcacat  
acatgttagc tgactgatga tggtaattt attttgycca tgggtgcaag  
tttctcttca ggaggaaaag cacagaactg gccaacaatt gcttgactgt  
tctttaccat actgttttagC AGGAAACCAr TCTCAGTGTC CCAACTCTCT  
AACCTTGGA CTTGTGAGAA CTCTGAGGAC AAAGCAGCGG ATACAACCTC  
AAAAGACGTC TGTCTACATT GAATTGGgta aggggtctcag gttttttaag  
tatttaataa taattgctgg attccttata ttatagtttt gccamaaatc  
ttggtcataa tttgtatttg tggtaggcag cwttggaag tgaattttat  
gagccctatg gtgagttata aaaaatgtaa aagacgcagt tcccaccttg  
aagaatctta ctttaaaaag ggagcaaaaag aggccaggca tgggtggctca  
cacctgtaat cccagcactt

SEQ ID NO:48 Exon 8 (BIC)

C AGGAAACCAr TCTCAGTGTC CCAACTCTCT AACCTTGGA CTTGTGAGAA  
CTCTGAGGAC AAAGCAGCGG ATACAACCTC AAAAGACGTC TGTCTACATT GAATTGG

## SEQ ID NO:49 Exon 15 and adjacent intronic sequences (BIC)

cagcctcccg agtagctgag attacaggcg ccagccacca caccagcta  
ctgacctgct tttvvvaaaac agctgggaga tatggtgcct cagaccatcc  
ccatgttata tgtcaaccct gacatattgg caggcaacat gaatccagac  
ttctaggctg tcatgcgggc tctttctttg ccagtcattn ctgactcttc  
tgacatgaac tgtntcaggt atgctttggc tgcccagcaa gtatgaaatg  
tcctntcaca aggggtggcg atgggttttct ccttccatth atctttctag  
GTCATCCCCT TCTAAATGCC CATCATTAGA TGATAGGTGG TACATGCACA  
GTTGCTCTGG GAGTCTTCAG AATAGAACT ACCCACCTCA AGAGGAGCTC  
ATTAAGGTTG TTGATGTGGA GGAGCAACAG CTGGAAGAGT CTGGGCCACA  
CGATTTGACG GAAACATCTT ACTTGCCAAG GCAAGATCTA Ggtaatatth  
catctgctgt attggarcam acactyrgat tttactctga atcctacata  
aagatatthct ggttanccaa cttttagatg trctagtcta tcatggacac  
ttttgttata cttaattaag cccactthtag aaaaatagct caagtgttaa  
tcwaggtthta cttgwaaatt attgaaactg ttaatccatc tatatthtaa  
ttaatggtht aactaatgat tttgaggatg wgggagctct ggtgtactct  
amatgtatta tttcaggcca ggcatagtgg ctcacgctg gtaatcccag  
tayycmrgag cccgaggcag gtggagccag ctgaggtcag

## SEQ ID NO:50 Exon 15 (BIC)

GTCATCCCCT TCTAAATGCC CATCATTAGA TGATAGGTGG TACATGCACA  
GTTGCTCTGG GAGTCTTCAG AATAGAACT ACCCACCTCA AGAGGAGCTC  
ATTAAGGTTG TTGATGTGGA GGAGCAACAG CTGGAAGAGT CTGGGCCACA  
CGATTTGACG GAAACATCTT ACTTGCCAAG GCAAGATCTA G

## SEQ ID NO:51 Exons 22 and 23 and adjacent intronic sequences (BIC)

tttgag agactatcaa accttatacc aagtggcctt atggagactg

ataaccagag tacatggcat atcagtggca aattgactta aaatccatac  
ccctactatt ttaagaccat tgtcctttgg agcagagaga cagactctcc  
cattgagagg tcttgctata agccttcac cggagagtgt agggtagagg  
gcctgggtta agtatgcaga ttactgcagt gattttacat ctaaagtcc  
atTTTTAGATC AACTGGAATG GATGGTACAG CTGTGTGGTG CTTCTGTGGT  
GAAGGAGCTT TCATCATTCA CCCTTGGCAC AGtaagtatt gggTgccctg  
tcagagaggg aggacacaat attctctcct gtgagcaaga ctggcacctg  
tcagtcacctc tggatgcccc tactgtagcc tcagaagtct tctcVVVVVc  
agagcaagac cctgtctcaa aaacaaacaa aaaaaatgat gaagtgcacg  
ttccagtagt cctactttga cactttgaat gctctttcct tcctggggan  
ccagGTGTCC ACCCAATTGT GGTGTGCAG CCAGATGCCT GGACAGAGGA  
CAATGGCTTC CATGtttatc actcattacc tggTgcttga gtagcacagt  
tcttggcaca tttttaaata tttgttgaat gaatggctaa aatgtctttt  
tgatgttttt attgttattt gttttatatt gtaaaagtaa tacatgaact  
gtttccatgg ggtgggagta agatatgaat

SEQ ID NO:52 **Exon 22 (BIC)**

ATC AACTGGAATG GATGGTACAG CTGTGTGGTG CTTCTGTGGT GAAGGAGCTT  
TCATCATTCA CCCTTGGCAC AG

SEQ ID NO:53 **Exon 23 (BIC)**

GTGTCC ACCCAATTGT GGTGTGCAG CCAGATGCCT GGACAGAGGA CAATGGCTTC  
CATG

SEQ ID NO: 54 **Forward primer of the invention for exon 8:**

GCA AAA TTC CTG GGC ATT

SEQ ID NO:55 Reverse primer of the invention for exon 8  
CAA AAA TCT TGG TCA TAA T

SEQ ID NO:56 Forward primer of the invention for exon 15  
GAT TTG TCC TTT CAC AAT TGG

SEQ ID NO:57 Reverse primer of the invention for exon 15  
GGA ACA AAC ACT TTG ATT TTA

SEQ ID NO:58 Forward primer of the invention for exon 18  
TTT GAG TGT TTT TCA TTC TG

SEQ ID NO:59 Reverse primer of the invention for exon 18  
GAG TCT GAG TTA CCA AAA GGT

SEQ ID NO:60 Forward primer of the invention for exon 20  
TGA TGG GTT GTG TTT GGT TT

SEQ ID NO:61 Reverse primer of the invention for exon 21  
AAC CCC AGA GTT CCA GCA C

Several pairs of forward and reverse primers for Exons 18, 20, 21, and 23 which are included in the invention are shown in Table 3. It is noted that the forward primers for Exons 20, 21 and 23 include a GC clamp of 40 bases.

Table 3

## FORWARD AND REVERSE PRIMERS OF THE INVENTION

Exon	Forward primer	Reverse primer
Exon 18	5' TTCAACTTCTAATCCTT -3'	5' CCCGCCGCGCCCCGCGCCCGTCCCGC GCCCCGCCCCGTGAGGTGTTAAAGGAGG A 3'
Exon 20	5' CGCCCGCCGCGCCCCGCGCCCGTCCCG CCGCCCCCGCCCCGTAAATATGACGTGTCT GCTC 3'	5' TGAAGCGGCCCATCTCTGCA 3'
Exon 21	5' CGCCCGCCGCGCCCCGCGCCCGTCCCG CCGCCCCCGCCCCGTATAACTAGATTTTCC TTCT 3'	5' CTCCACTATGTAAGACAA 3'
Exon 23	5' CGCCCGCCGCGCCCCGCGCCCGTCCCG CCGCCCCCGCCCCGTGATGAAGTGACAGTT CCAG 3'	5' GTGATAAACCAAACCCATGC 3'

Additional primers of the invention which should be useful for CDGE and other applications are shown in Table 4.

Table 4

---

ADDITIONAL FORWARD AND REVERSE PRIMERS OF THE INVENTION	
<u>Exon/Direction*</u>	<u>Primer</u>
8F	5'-GCA AAA TTC CTG GGC ATT-3' (SEQ ID NO:54)
8R	5'-CAA AAA TCT TGG TCA TAA T-3' (SEQ ID NO:55)
15F	5'-GAT TTG TCC TTT CAC AAT TGG-3' (SEQ ID NO:56)
15R	5'-GGA ACA AAC ACT TTG ATT TTA-3' (SEQ ID NO:57)
18F	5'-TTT GAG TGT TTT TCA TTC TG-3' (SEQ ID NO:58)
18R	5'-GAG TCT GAG TTA CCA AAA GGT-3' (SEQ ID NO:59)
20F	5'-TGA TGG GTT GTG TTT GGT TT-3' (SEQ ID NO:60)
21R	5'-AAC CCC AGA GTT CCA GCA C-3' (SEQ ID NO:61)

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\*"F" designates forward primer, "R" designates reverse primer

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While the invention has been described in connection with what is presently considered to be the most practical and preferred embodiments, it is to be understood that the invention is not to be limited to the disclosed embodiments, but is intended to cover various modifications included within the spirit and scope of the appended claims.

References cited herein are hereby incorporated by reference.



What is claimed is:

1. An isolated DNA sequence comprising SEQ ID NO:34.
2. An isolated DNA sequence, comprising:
  - a) SEQ ID NO:10, or a sequence which is at least 95% identical thereto; and
  - b) Exon 20, or a sequence which is at least 95% identical thereto.
3. The DNA sequence of claim 2 which additionally comprises an 83 bases pair sequence downstream of Exon 20.
4. An isolated DNA sequence comprising SEQ ID NO:10, or a fragment of at least 15 contiguous bases thereof.
5. The sequence of claim 4 which is at least 20 contiguous bases of SEQ ID NO:10.
6. The DNA sequence of claim 4 which additionally comprises Exon 20 of the BRCA1.
7. The DNA sequence of claim 6 which additionally comprises at least 15 contiguous bases of SEQ ID NO:35.
8. An isolated DNA sequence, comprising:  
CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCGTAAATATGACGTGTCTGCTCCA  
CTTCCATTGAAGGAAGCTTCTCTTTCTCTTATCCTGATGGGTTGTGTTTGGTTTCTTTCAGC

ATGATTTTGAAGTCAGAGGAGATGTGGTCAATGGAAGAAACCACCAAGGTCCAAAGCGAGCA  
AGAGAATCCCAGGACAGAAAGGTAAAGCTCCCTCCCTCAAGTTGACAAAAATCTCACCAC  
CACTCTGTATTCCACTCCCCTTTGCAGAGATGGGCCGCTTCA (SEQ ID NO:36), or  
a sequence which is at least 95% identical thereto.

9. An isolated oligonucleotide primer which specifically hybridizes to human chromosome 17, said primer comprising SEQ ID NO:31 or a sequence which is at least 90% identical thereto, and said primer being capable of directing amplification of exon 20 of the BRCA1 gene.
10. The primer of claim 9 which comprises a sequence at least 95% identical to SEQ ID NO:31.
11. The primer of claim 9 which additionally comprises a sequence which is a GC clamp.
12. The primer of claim 11 wherein said GC clamp comprises SEQ ID NO:37.
13. The primer of claim 12 which is SEQ ID NO:32.
14. The primer of claim 9 which is labeled with a radiolabel, a fluorescent label, a bioluminescent label, a chemiluminescent label, or an enzyme label.
15. An isolated oligonucleotide primer which specifically hybridizes to human chromosome 17, said primer comprising SEQ

ID NO:33 or a sequence which is at least 90% identical thereto.

16. The primer of claim 15 which comprises a sequence at least 95% identical to SEQ ID NO:33.

17. An isolated oligonucleotide primer according to claim 15 which is labeled with a radiolabel, a fluorescent label, a bioluminescent label, a chemiluminescent label, or an enzyme label.

18. An isolated oligonucleotide sequence comprising exon 20 of the BRCA1 gene or a substantially similar sequence which has been amplified by a primer according to claim 15.

19. A method of making exon 20 of the BRCA1 gene or a substantially similar sequence, comprising:

- (a) exposing a primer according to claim 9 to bases in the presence of an amplifying enzyme; and
- (b) allowing amplification to take place.

20. The method of claim 19 additionally comprising exposing a primer according to claim 15 to bases in the presence of amplifying enzyme.

21. A method of determining the presence or absence of a mutation in exon 20 of the BRCA1 gene, comprising:

- (a) exposing a primer according to claim 9 to a DNA

sample containing the BRCA1 gene in the presence of an amplifying enzyme, and nucleoside bases;

(b) allowing the primer(s) to direct amplification of exon 20, thereby obtaining an amplified product;

(c) comparing the sequence of the product to the normal sequence of exon 20 by using CDGE to determine the presence or absence of an aberrant migration pattern; and

(d) if an aberrant migration pattern is present, sequencing the product to determine whether a mutation or a normal polymorphism is present.

22. A method of determining a predisposition or higher susceptibility to breast or ovarian cancer in a person based on the presence of a mutation in exon 20 of the BRCA1 gene, comprising:

(a) exposing a primer according to claim 9 to a DNA sample containing the BRCA1 gene in the presence of an amplifying enzyme, and nucleoside bases;

(b) allowing the primer(s) to direct amplification of exon 20;

(c) comparing the sequence of the product to the normal sequence of exon 20 by using CDGE to determine the presence or absence of an aberrant migration pattern;

(d) if an aberrant migration pattern is present, sequencing the product to determine whether a mutation or a normal polymorphism is present; and

(e) determining from the presence of the mutation that the person has a predisposition or higher susceptibility to

breast or ovarian cancer.

23. An isolated DNA sequence comprising SEQ ID NO:7 or SEQ ID NO:9.

24. An isolated oligonucleotide primer comprising a sequence selected from the group consisting of SEQ ID NOS:41-46 and 54-61.

25. An isolated oligonucleotide primer which specifically hybridizes to human chromosome 17, said primer comprising a sequence selected from the group consisting of SEQ ID NOS:41-42 or a sequence which is at least 90% identical thereto.

26. An isolated oligonucleotide sequence comprising exon 21 of the BRCA1 gene or a substantially similar sequence which has been amplified by a primer according to claim 25.

27. A method of determining the presence or absence of a mutation in exon 21 of the BRCA1 gene, comprising:

(a) exposing a primer according to claim 25 to a DNA sample containing the BRCA1 gene in the presence of an amplifying enzyme, and nucleoside bases;

(b) allowing the primer(s) to direct amplification of exon 21;

(c) comparing the sequence of the product to the normal sequence of exon 21 by using CDGE to determine the presence or absence of an aberrant migration pattern; and

(d) if an aberrant migration pattern is present, sequencing the product to determine whether a mutation or a normal polymorphism is present.

28. A method of determining a predisposition or higher susceptibility to breast or ovarian cancer in a person based on the presence of a mutation in exon 21 of the BRCA1 gene, comprising:

(a) exposing a primer according to claim 25 to a DNA sample containing the BRCA1 gene in the presence of an amplifying enzyme, and nucleoside bases;

(b) allowing the primer(s) to direct amplification of exon 21;

(c) comparing the sequence of the product to the normal sequence of exon 21 by using CDGE to determine the presence or absence of an aberrant migration pattern;

(d) if an aberrant migration pattern is present, sequencing the product to determine whether a mutation or a normal polymorphism is present; and

(e) determining from the presence of the mutation that the person has a predisposition or higher susceptibility to breast or ovarian cancer.

29. An isolated oligonucleotide sequence comprising exon 8, exon 15, exon 18, exon 20, exon 21 or exon 23 of the BRCA1 gene or a substantially similar sequence, which has been amplified by a primer according to claim 24.

30. An isolated DNA sequence, comprising:

a) SEQ ID NO:16, or a sequence which is at least 95% identical thereto; and

b) Exon 15, or a sequence which is at least 95% identical thereto.

31. An isolated DNA sequence, comprising:

a) SEQ ID NO:18, or a sequence which is at least 95% identical thereto; and

b) Exon 15, or a sequence which is at least 95% identical thereto.

32. An isolated DNA sequence, comprising:

a) SEQ ID NO:20, or a sequence which is at least 95% identical thereto; and

b) Exon 18, or a sequence which is at least 95% identical thereto.

33. An isolated DNA sequence, comprising:

a) SEQ ID NO:22, or a sequence which is at least 95% identical thereto; and

b) Exon 18, or a sequence which is at least 95% identical thereto.

34. An isolated DNA sequence, comprising:

a) SEQ ID NO:25, or a sequence which is at least 95% identical thereto; and

b) Exon 21, or a sequence which is at least 95%

identical thereto.

35. An isolated DNA sequence, comprising:

- a) SEQ ID NO:28, or a sequence which is at least 95% identical thereto; and
- b) Exon 23, or a sequence which is at least 95% identical thereto.

36. An isolated DNA sequence, comprising:

- a) SEQ ID NO:29, or a sequence which is at least 95% identical thereto; and
- b) Exon 23, or a sequence which is at least 95% identical thereto.

37. An isolated nucleotide sequence comprising at least one of SEQ ID NOS: 4, 7, 9, 10, 12, 14, 16, 18, 20, 22, 24, 25, 28, 29, 31, 32, 33, 34, 36, 41-46 and 54-61.



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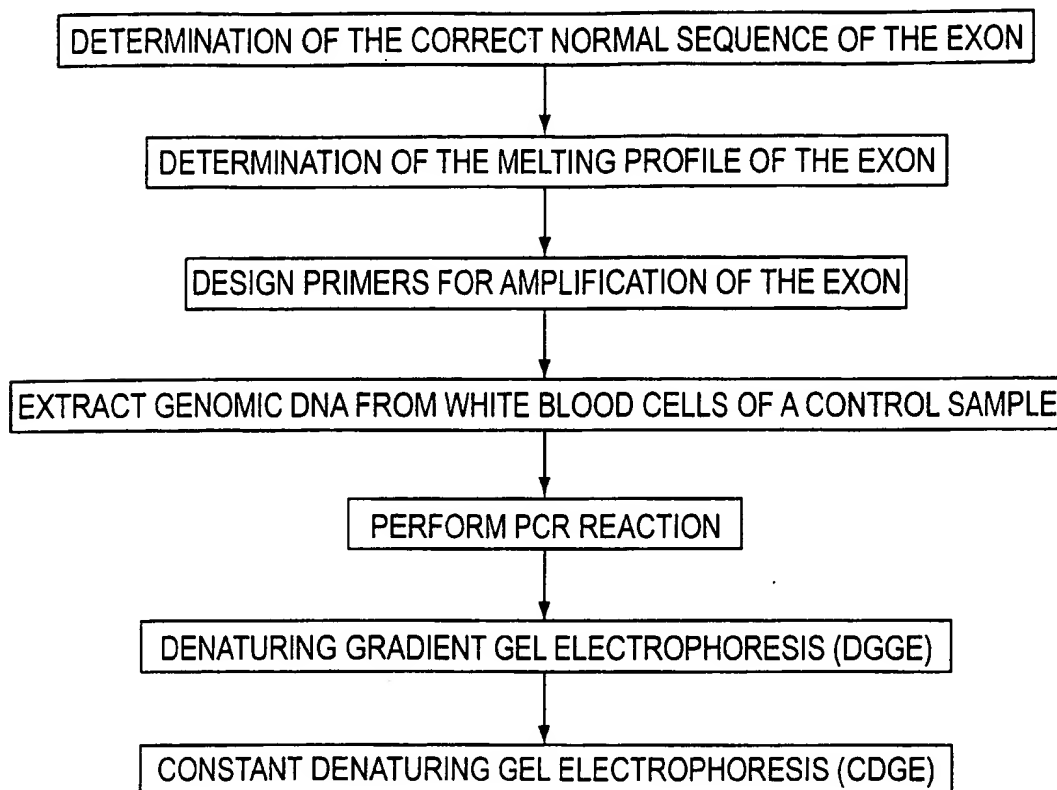
OPTIMIZATION STRATEGY FOR CDGE MUTATION ANALYSIS OF A BRCA 1 EXON

FIG. 1A

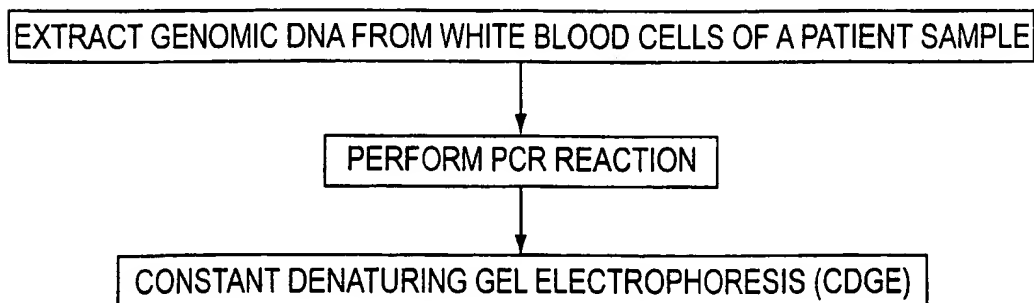
STANDARD STRATEGY FOR CDGE MUTATION ANALYSIS OF A BRCA 1 EXON

FIG. 1B

1 CGCCCGCCG GCGCCGCGCC CGTCCCGCCG CCGCCGCGCC TAAATATGAC  
 51 GTGTCTGCTC CACTTCCATT GAAGGAAGCT TCTCTTTCTC TTATCCTGAT  
 101 GGGTTGTGTT TGGTTTCTTT CAGCATGATT TTGAAGTCAG AGGAGATGTG  
 151 GTCAATGGAA GAAACCAACA AGGTCCAAAG CGAGCAAGAG AATCCCAGGA  
 201 CAGAAAGGTA AAGCTCCCTC CCTCAAGTTG AAAAAAATCT CACCCACCA  
 251 CTCTGTATTC CACTCCCTTT TGCAGAGATG GGCCGCTTCA

FIG. 2A

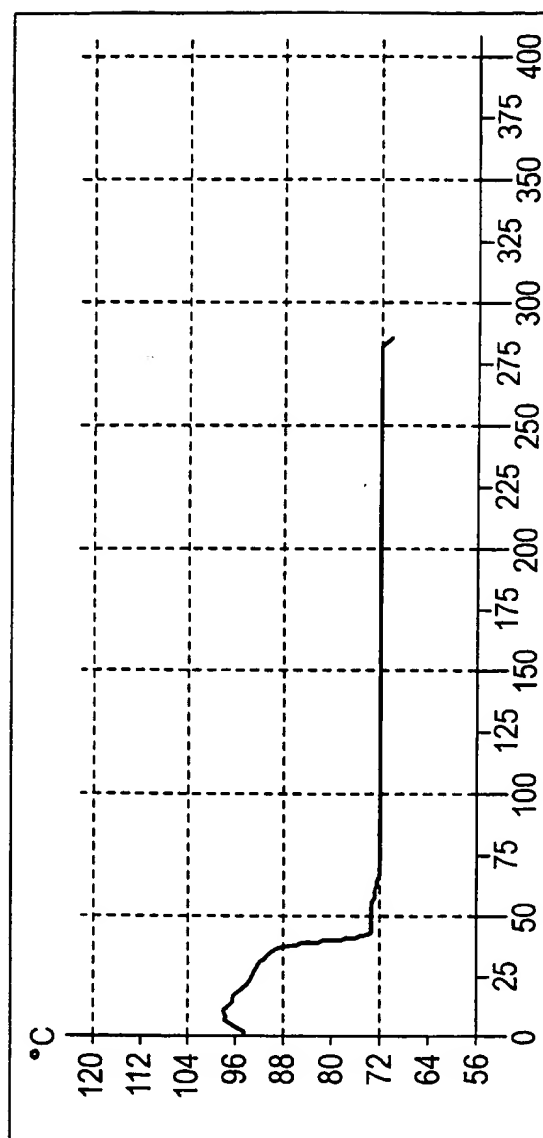


FIG. 2B

1 CGCCCGCCGC GCCCGCGGCC CGTCCCGCCG CCCCCGCCG ATATGACGTG  
 51 TCTGCTCCAC TTCCATTGAA GGAAGCTTCT CTTTCTCTTA TCCTGATGGG  
 101 TTGTGTTTGG TTTCTTTTCAG CATGATTTTG AAGTCAGAGG AGATGTGGTC  
 151 AATGGAAGAA ACCACCAAGG TCCAAAGCGA GCAAGAGAAT CCCAGGACAG  
 201 AAAGGTAAAG CTCCCTCCCT CAAGTTGACA AAAATCTCAC CCCACCACTC  
 251 TGTATTCCAC TCCCTTTTGC AGAGATGGGC CGCTTCATT TTGTAAGACT

FIG. 3A

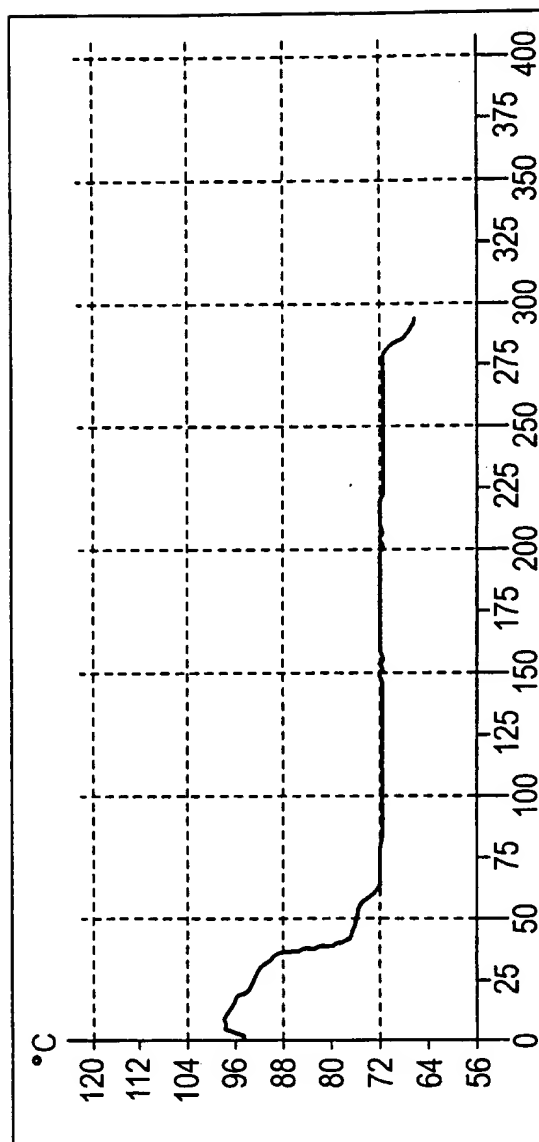


FIG. 3B

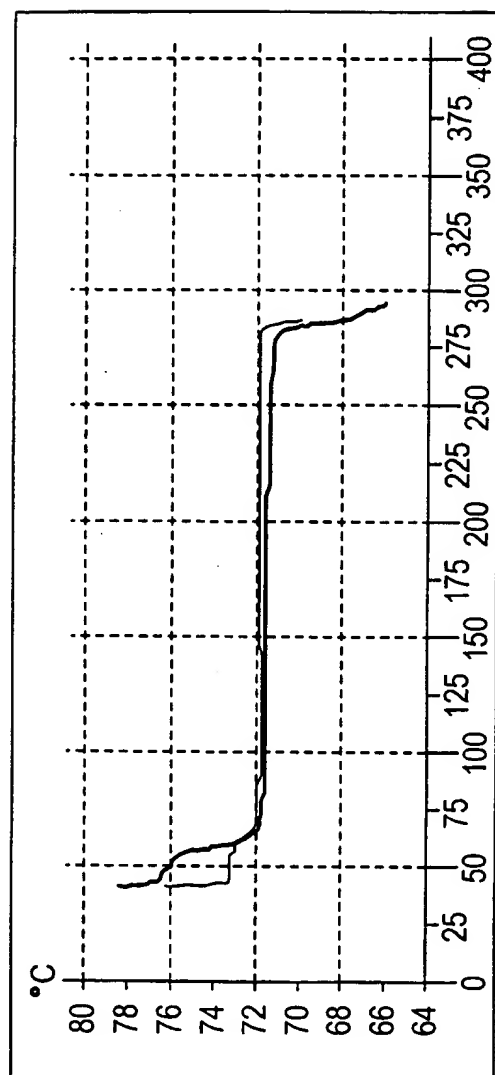


FIG. 4

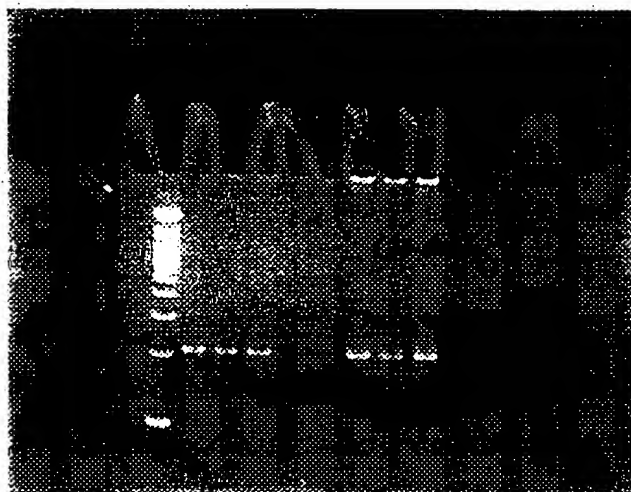


FIG. 5

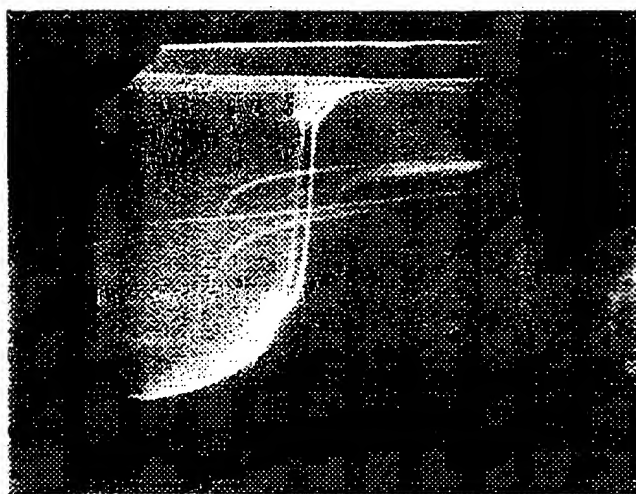


FIG. 6

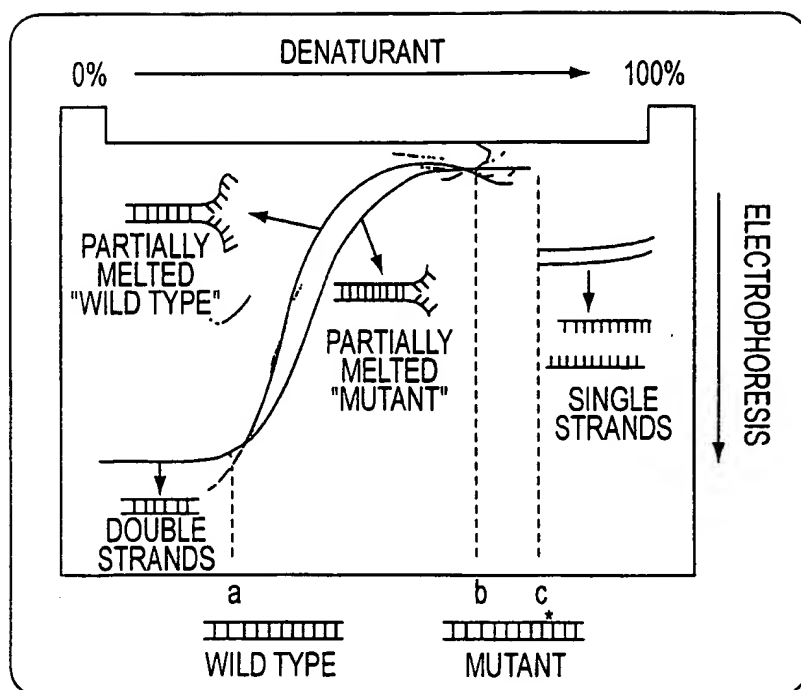


FIG. 7

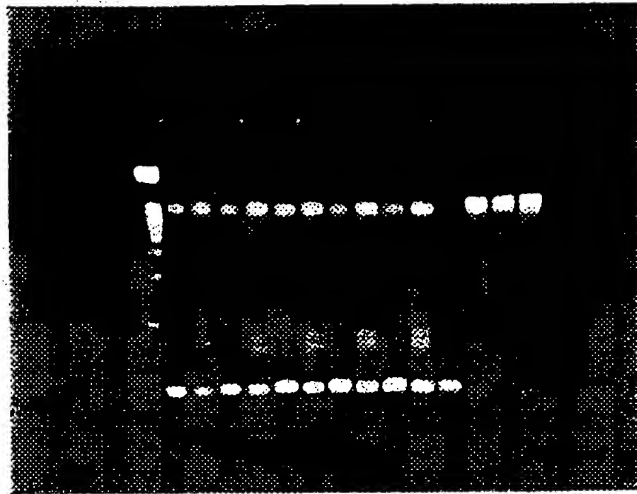


FIG. 8



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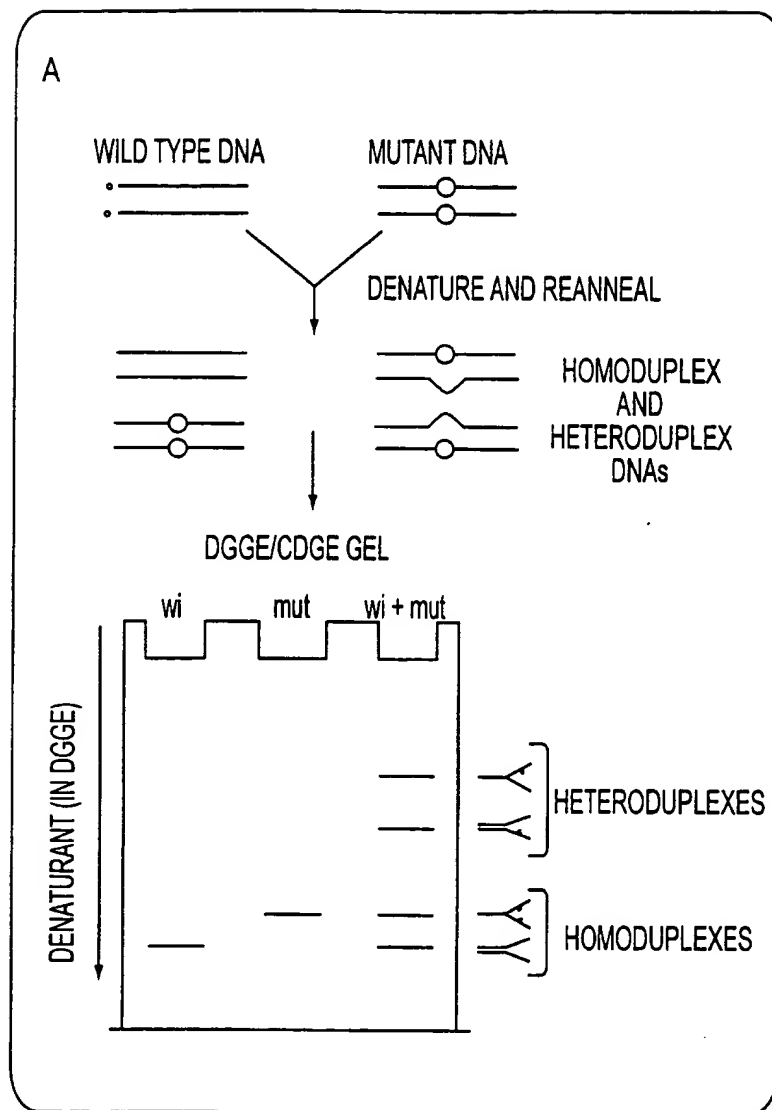


FIG. 9

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/19596**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : C12Q 1/68; C12P 19/34; C07H 21/04

US CL : 435/6, 91.2; 536/24.31, 24.33

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.2; 536/24.31, 24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS; DIALOG: Medline, CA, Derwent Patents, Biosis, EMBASE:

search terms: BRCA1, probe, primer, mutation, consensus sequence, conserved sequence, mutation, exon

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SHATTUCK-EIDENS et al. A collaborative survey of 80 mutations in the <i>BRCA1</i> breast and ovarian cancer susceptibility gene. Journal of the American Medical Association. 15 February 1995, Vol. 273, No. 7, pages 535-541, especially page 538.	2, 3, 18, 31-37
Y, P	US 5,654,155 A (P.D. MURPHY et al) 05 August 1997, column 1, Table II.	2, 3, 18, 31-37
Y, E	US 5,693,473 A (SHATTUCK-EIDENS et al) 02 December 1997, columns 64-69.	2, 3, 18, 31-37
Y, P	US 5,622,829 A (KING et al) 22 April 1997, Table 1, columns 9-11.	2, 3, 18, 31-37

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

06 JANUARY 1998

Date of mailing of the international search report

06 FEB 1998

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

CARLA MYERS

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/19596**Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☒ Claims Nos.: 1, 4-17, 19-30, 38  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
  
Please See Extra Sheet.
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/19596

## BOX I. OBSERVATIONS WHERE CLAIMS WERE FOUND UNSEARCHABLE

### 2. Where no meaningful search could be carried out, specifically:

The claims are unsearchable to the extent that they require the search of a specific nucleotide sequence. Because no computer readable copy of the sequence has been provided, a meaningful search of the recited sequences cannot be carried out by this Authority. However, the subject matter of the claims has been searched to the extent possible with reference to the balance of the description.